REMARKS

Claims 18, 20-30, and 33-35 are pending in this application. Claims 1-17 and 19 have been cancelled. Claims 33 and 34 have been rejoined. Claim 35 has been amended to insert a period at the end of the claim. Claims 31 and 32 are withdrawn. It is respectfully requested that claims 31 and 32 be rejoined in this application.

Claim 18 is amended to include in step b) treatment with only a single lysis solution. Support for this amendment is found throughout the specification and at page 8, line 2.

The Examiner considers that claims 18-30 and 33-35 are obvious over Fernandez et al. (hereinafter "D1") in view of Bezanehtak et al. (hereinafter "D2"). This is respectfully traversed.

The present invention provides for an improved and more reliable method for unambiguously detecting the fragmentation of chromatin in sperm based on a single lysis step with a single lysis solution that does not contain protein denaturing agents.

D1, as discussed in the background section of this application (see pages 3 and 4), describes a method for establishing DNA fragmentation of sperm cells. In a first step, a suspension containing sperm, embedded in an agarose microgel, is subject to a DNA denaturation step by using an acid denaturing solution (0.08 N HCI). Then, the proteins are removed by a lysis solution (solution 1) that comprises 0.4 M Tris, 0.8 MDDT, 1 % SDS and 50 mM EDTA, at pH 7.5. Then, incubation is performed with another lysis solution (solution 2) that comprises 0.4 M Tris, 2 M NaCI, and 1 % SDS at pH 7.5. Finally, the preparations obtained are subject to staining with DAPI for determination of fragmentation by fluorescence and microscopy of

bright field. D1 describes a method for the evaluation of DNA fragmentation in semen comprising a denaturation step of the sperm sample, a double lysing step with two solutions containing denaturing agents, such as SDS, and finally evaluation of the integrity of chromatin based on the measurement of the halo size of the sperm.

Claim 18 differs from document D1 in the following technical features:

- a single lysis step, instead of two sequential ones, with a single lysis solution, and
- the absence of denaturing detergents (SDS) in the single lysis solution.

These features are not disclosed in D1. Therefore, the method of claim 18 differs from D1 in that the lysis step is a single step and only a single lysis solution without denaturing agents is used. This has the effect of not affecting negatively the structure of the spermatozoids allowing for an unambiguous identification of these kind of cells with respect to other types of cells that may be present in the sample. The use of a single lysis step, together with not using denaturing agents in the lysis solution, also has the advantage that quality and contrast of the images obtained is very much improved giving rise to more reliable results with improved reproducibility.

As demonstrated in the application, and in Figures 3 and 4, a single lysis step and use of a single lysis solution that does not include a non-denaturing agent permits a much less aggressive treatment, which results in keeping the tails of the sperm cells connected to the cells. Keeping the tails is a critical improvement, as their identification is essential morphological data to distinguish the images of nucleoids in determining whether the nucleoids are those of sperm cells or nucleoids of other cells types that could be present, for instance, desquamated cells of the genitourinary tract, blood cells, etc. This, as shown in Figure 1, does not occur in the method of D1, and therefore, the method of D1 could not be

used commercially.

In addition, improvement of the claimed invention is in the quality of images of nucleoids that can be viewed with the necessary precision using a conventional bright field microscope. In document D1 the images of the nucleoids of sperm cells are stained with a fluorescent DNA dye (DAPI), and a fluorescence microscope should be available for viewing them. However, the single and milder lysis of the invention herein achieves unfolding of the chromatin loops maintaining the morphology of the previous head, or "core", and obtaining dispersion halos with a greater density of chromatin material. This enables halos, to be viewed under a conventional bright field microscope. Consequently, the contrast and visual discrimination of the different sizes of halos is much improved. Stainings, such as Diff-Quik, described in article D1, do not achieve an adequate contrast of the halo from the background when the technique is performed with a lysing solution with protein-denaturing detergent. Consequently, when the halo is greatly scattered, it is usually difficult to see its peripheral edge, that can be considered as a small halo, thus assigning the category of fragmented to a sperm cell with an unchanged DNA. Therefore, the procedure published in D1 tends to overestimate the fragmentation levels, particularly in bright field staining, resulting in a test that is not reliable. This improvement of the invention is extremely relevant for the reliability of the technique and results.

The use of a single lysis solution, in absence of SDS or another denaturing agent , allows for the possible sequential use in nucleoids, of protein immunodetection techniques, such as laminins and other nuclear proteins, and the detection of RNA associated with the nuclear matrix, as the DNA spreads maintaining the greatest amount possible of nuclear structure. This is important for research on the structure of the sperm cell nucleus and for diagnosis and

identification of medical conditions.

Finally, the single lysis step of this invention allows for using smaller amounts of reagents with the attendant lower costs. DTT is particularly costly and the reduction to the concentration described (a fourth of what is described in D1) is enough.

From the above, it is concluded that the invention provides images of sperm cell nucleoids that are much improved and much more reproducible, as compared to the protocol described in article D1 (as shown comparing Figure 2 to Figures 3 and 4). In the claimed invention, nucleoids from immature or mature sperm cells or other cell types can be distinguished, and the categorization of the halo size is much more precise. Consequently, with the procedure of the invention, the determination of DNA fragmentation levels of the sample is much more reliable than the procedure of D1. This is very important in developing and carrying out a test for clinical application to patients.

The Examiner's attention is also drawn to Figures 1, 2, 3, and 4 that accompany this response.

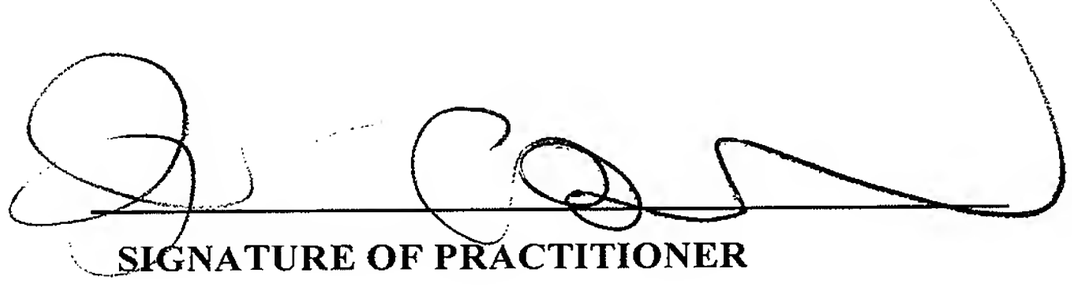
D2 teaches the effect of non ionic detergent, Triton X, on sperm demembranating and DNA condensation. However, D2 does not mention or suggest that a single step of lysis with a single lysing solution without denaturing agents would allow the conservation of the entire structure of the spermatozoids.

Therefore, starting from D1, and in view of D2, a skilled person in the art would have developed, in any case, a method based on the use of two sequential lysis steps, with two lysing solutions, with or without denaturing agents, with the disadvantages that this involves. But, in no case, would someone skilled in the art have developed a method as that of the

present invention, with a single step lysis (a single lysis solution), in the light of these two documents. There is no combination of the documents that discloses, teaches, or suggests the claimed invention. Therefore, it is respectfully requested that this rejection be withdrawn

Accordingly, it is submitted that this application is in condition for allowance and favorable consideration is respectfully requested.

If any fees are due, please charge deposit account 12-0425.



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